REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1999	Annual (1 Sep 98 - 31 Aug 99)

4. TITLE AND SUBTITLE
Role of MYC in Anchorage-Dependent Growth

5. FUNDING NUMBERS
DAMD17-97-1-7015

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release ;
distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

When deprived of adhesion to solid substrate, non-hematopoietic, normal cells are unable to proliferate; instead, they arrest in the G1 phase of the cell cycle. Unlike normal cells, transformed cells are able to bypass this integrin-mediated control of proliferation and to grow in an anchorage-independent manner. Understanding the mechanism by which the extracellular matrix controls progression through the G1 phase of the cell cycle would give us insight into cellular alterations that accompany carcinogenesis. We have shown, here using a mammary epithelial model, that the expression of the early growth control gene, c-Myc, is directly regulated by cell adhesion through a β1 integrin-dependent pathway. When deprived of adhesion, mammary epithelial cells are unable to progress into S phase upon EGF stimulation, and they arrest in early G1. This anchorage-dependent G1 block correlates with the downregulation of c-Myc mRNA and protein. Overexpression of c-Myc in these cells overcomes the G1 block, allowing for and anchorage independent downregulation of p27, activation of cyclin E-CDK2, and progression into S phase.
Our study shows that c-myc is a crucial player in mediating anchorage-dependent control of cell cycle progression in human mammary epithelial cells.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer anchorage-dependence, c-Myc, HMEC, cell cycle, integrin	16
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

AD_____

Award Number: DAMD17-97-1-7015

TITLE: **Role of MYC in Anchorage-Dependent Growth**

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REPORT DATE: September 1999

TYPE OF REPORT: **Annual**

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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C Benaud 9/01/99

PI - ~~Signature~~ Date

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Introduction:

To proliferate, normal cells require two types of extracellular signals: hormonal/growth factor stimuli and adhesion to the extracellular matrix (ECM). When deprived of attachment to a solid substrate, even in the presence of growth factors, normal cells are unable to replicate their DNA, and they arrest in the G1 phase of the cell cycle (1,2). A hallmark of transformed cells, which correlates with tumorigenicity, is the loss of anchorage-dependent growth. As cells become malignant, they gain the ability to bypass the extracellular matrix control of cell proliferation.

Recent studies have linked cell adhesion to key regulatory molecules of cell cycle progression: the cyclins and their associated cyclin-dependent kinases (cdk). In fibroblasts, loss of adhesion results in a downregulation of cyclins D and A, and an accumulation of the CDK inhibitors, p21 and p27, concurrent with an inhibition of the cyclin E-CDK2 activity (1,3,4,5).

A potential and still controversial link between integrin-mediated cell adhesion and upregulation of cyclin expression and cdk activity is the c-Myc proto-oncogene, which is frequently overexpressed in breast cancer. Experiments altering Myc expression indicate that Myc is required for cell proliferation and that it plays a crucial role in the progression through the G1 phase of the cell cycle (6,7,8). Myc has been shown to induce cyclin E and cyclin A expression (9), as well as the activity of the cyclin E-cdk2 complex, presumably by triggering the release of the kinase inhibitors p27 and p21 (10,11), an effect strongly reminiscent of the one caused by cell adhesion.

Studies of the signaling pathways from integrins to the cell cycle machinery have been concentrating on the ones leading to upregulation of the c-Jun and c-Fos immediate early response genes, explaining the anchorage-dependent expression of cyclin D (12). Possible signaling pathways regulating c-Myc expression and function have been, to a large extent, unexplored. The regulation of c-Myc by cell adhesion seems to be cell type-specific. For

example, the c-Myc mRNA has been reported to be down regulated by lack of adhesion in endothelial cells (13), however, in fibroblasts this adhesion-dependent regulation does not appear to occur, or is only a secondary event(1,14). To date, the role of integrins in the regulation of c-Myc expression and function has not been explored in epithelial systems.

We have thus proposed to study whether in human mammary epithelial cells, c-Myc represents a link between ECM adhesion-dependent growth and the cell cycle regulatory machinery.

Body:

During the September 97- August 98 period we had addressed Aim 2 of the original proposal:

Aim 2: To characterize the role of Myc in mediating anchorage-dependent regulation of cyclin-dependent kinase activity.

a. Investigate whether cell anchorage activates two parallel pathways, one Myc-independent leading to the expression of cyclin D1, the second involving Myc activation of cyclin E-cdk2 activity.

b. Define how Myc induces cdk2 activation, focusing on cyclin E expression, p21 and p27 expression, inhibitor association with the cyclin-cdk2 complex, and regulation of cdc25 phosphatase.

As the first step toward approaching this aim, we first wanted to establish whether deregulated expression of c-Myc enables mammary epithelial cells to progress through the anchorage-dependent G1 checkpoint. Specifically, we wanted to determine if this c-Myc induced G1-S transition is achieved through the activation of G1 cyclin-CDK complex due to the down regulation of CDK inhibitors.

We had previously shown, using flow cytometry, that the immortalized mammary epithelial cell line 184AIN4 (15), can be synchronized and growth arrested in the G0/G1 stage of the cell cycle by EGF withdrawal. Upon readdition of EGF, cells in monolayer progress

into S and G2 phases of the cell cycle. However, cells stimulated with EGF in suspension culture were unable to enter S phase. In contrast, A1N4 cells stably transfected with c-Myc under a constitutive promoter (A1N4-Myc cells) (16) are able to replicate their DNA in suspension culture. The overriding of the adhesion-dependent G1 check point by overexpression of c-Myc was further supported at the molecular level by studies of the phosphorylation state of the retinoblastoma protein, pRb. Whereas pRb could only be detected in its hypophosphorylated form in non-adherent A1N4 cells, hyperphosphorylated forms of pRb are present in the Myc transfectants. These results confirm that A1N4 myc cells progress through the G1-S transition in the absence of adhesion (Data shown in the previous Annual Report).

We then asked whether Myc is able to induce the expression and activity of the various G1 cyclin-CDK complexes in an adhesion-independent manner. Our results indicated that low levels of cyclin D-cdk4 activity were still present in non-adherent A1N4 cells and that overexpression of c-Myc does not upregulate CDK4 activity. We then examined the cyclin E-CDK2 complex. Whereas lack of adhesion completely inhibited CDK2 kinase activity, overexpression of c-Myc restored CDK2 activity to similar levels that those detected in adherent cells. Unlike the parental cells that only express very low levels of cyclin E in suspension, A1N4-myc cells are able to upregulate cyclin E in an adhesion-independent manner. In addition, the phosphorylated form of CDK2, which corresponds to the active form of the kinase, was present in non-adherent A1N4-Myc cells, but was not detectable in the parental cells. This observation confirms the difference in CDK2 kinase activity detected by *in vitro* kinase assay. This difference in kinase activity could be explained by the observation that the levels of the CDK2 inhibitor, p27 were drastically changed between the parental cells and the transfectants. Whereas in non-adherent parental A1N4 cells p27 was present at high levels and found complexed with CDK2, p27 was not detectable in c-Myc overexpressing cells. We

have further shown that p27 is not downregulated by a decrease in transcription but by a decrease in the stability of the protein which is rapidly degraded by the ubiquitin-proteosome pathway in non-adherent A1N4-Myc cells (Data shown in the previous Annual Report).

We have now in the September 98- August 99 period addressed Aim 1 of the original proposal:

Aim 1: To determine whether cell adhesion to ECM components is required for Myc induction and activation.

a. Analyze whether cell adhesion alters Myc expression, more specifically Myc mRNA levels, mRNA stability, and protein levels.

b. Study the effect of cell anchorage on Myc activity. The focus will be on adhesion-dependent alteration of Myc phosphorylation, p107 association with Myc, as well as phosphorylation and expression levels of Max.

c. If cell adhesion activates Myc, we will define whether particular ligands and specific members of the β1 family of integrins mediate the regulation of Myc in epithelial cells.

Following the proposal in Aim 1-a (months 1-6) we first used western analysis to examine the levels of c-myc protein in adherent and non-adherent mammary epithelial cells lines (figure1A). As previously described in fibroblasts, when quiescent A1N4 cells, which have been maintained for 60-72 hrs in low serum without EGF, are stimulated under adherent condition with EGF, a strong increase in c-Myc protein levels can be observed. c-Myc increases within one hour, peaking by two hours, and then decreases slightly but is maintained at an elevated level throughout the rest of G1 phase. Instead, if A1N4 cells are stimulated with EGF in suspension, a weaker initial increase in c-Myc can be detected. However, by 4 hrs of suspension culture, Myc becomes undetectable by western blot, indicating a strong downregulation of c-Myc levels in response to the lack of adhesion. The direct effect of adhesion on c-Myc levels has been confirmed by transferring cells maintained in suspension for 4 hrs back to regular tissue

culture dishes for reattachment (figure 1B). Under these conditions, c-Myc protein levels start to increase within two hours of readhesion, and by 4hrs c-Myc expression levels reach levels comparable to those detected in cells grown in monolayer. To ensure that the regulation of c-Myc by adhesion is not a particularity of the A1N4 cell line, we looked at another non-transformed mammary epithelial cell line, MCF-10A (figure 1C). Similar to our observations in the A1N4, when deprived of adhesion, the MCF-10A also downregulate their level of c-Myc protein within two hours, and c-Myc becomes undetectable after 6 hrs of suspension culture. We thus asked whether this downregulation of c-Myc occurs at the mRNA level (figure1D). Northern analysis examining steady state levels of c-Myc mRNA, shows that the initial increase of Myc mRNA following EGF stimulation occurs both in adhrent and non-adherent cells. However, after four hours a three-fold decrease can be observed in mRNA levels in non-adherent cells as compared to adherent cells. These results suggest that cell adhesion is required for maximally sustained expression of c-Myc in epithelial cells. Treatment with cycloheximide can reverse the downregulation of c-Myc mRNA in suspended cells, indicating that protein synthesis or a labile protein is required for c-Myc mRNA downregulation. To try to differentiate between a transcriptional event or a decrease in messanger RNA stability, we treated non-adherent A1N4 cells for up to 120 min with Actinomcin D (figure 1E). Northern blot for c-Myc mRNA showed only a slight decrease in the stability of c-Myc mRNA in these non-adherent cells. An increase of mRNA turnover is, therefore, not the main mechanism of Myc mRNA downregulation, suggesting a transcriptional regulation of the c-*myc* gene.

Since the c-Myc protein is downregulated to undetectable level when mammary epithelial cells are deprived of adhesion, we decided not to address part b of Aim 1 that proposed to investigate a modulation in Myc function, and instead, we addressed directly

Aim1c (month 19-24). To examine whether specific ECM components can modulate c-Myc expression, we stimulated cells with EGF in suspension culture for 4 hour and then transferred the cells to either tissue culture dishes or petri dishes coated with fibronectin (figure 2A). Western blot analysis showed that the upregulation of the c-Myc protein, following reattachment, is enhanced in cells plated onto fibronectin. We then asked whether adhesion to ECM components only allows for optimal EGF signaling or whether an integrin signaling pathway is cooperating with the EGF pathway. To address the latest possibility, we tested whether adhesion to ECM components in the absence of growth factors can induce c-Myc expression. When resting cells are transferred and allowed to attach to fibronectin-coated dishes, in the absence of growth factor addition, a transient upregulation of Myc protein starting at 30min and peaking at 1-2 hours can be detected by western blot (figure2B). Furthermore, this induction of c-Myc is fibronectin-density dependent (figure 2C). A similar upregulation of c-Myc can be observed when cells are plated on collagen, but not laminin. Fibronectin induction of c-Myc requires an intact cytoskeleton, since disruption of the actin cytoskeleton by Cytochalasin D treatment prevents c-Myc expression. Addition of fibronectin in suspension does not induce c-Myc expression. Instead, incubation of cells in suspension with fibronectin-coated microbeads, that allows for a solid support for integrin aggregation, leads to an increase in c-Myc levels. In contrast, control BSA-coated beads do not lead to c-Myc induction. Taken together these results suggest a role for integrin signaling in the upregulation of c-Myc by fibronectin. To prove this, we used a blocking antibody against β1 integrin (figure 2D). Plating the cells onto fibronectin in the presence of β1 blocking antibody impedes the upregulation of c-Myc in an antibody-concentration dependent manner, directly implicating β1 integrin in the regulation of c-Myc by fibronectin.

We are now proposing to identify which specific β1 integrin dimer is responsible

for the regulation of c-Myc. We have started to examine by flow cytometry which fibronectin-binding integrins are present at the cell surface of the A1N4. We then propose to identify the signaling molecules responsible for transmitting the regulatory message from the integrin to c-Myc.

Key research accomplishments:

We have shown that:

- In human mammary epithelial cell adhesion is required for sustained c-Myc expression both at the messenger RNA and protein level
- Adhesion to ECM components enhances growth factor dependent induction of c-Myc
- Independent of growth factors, β1 integrin can induce c-Myc expression
- In the absence of adhesion, human mammary epithelial cells arrest in early G1
- Deregulated expression of c-Myc enable A1N4 cells to bypass the anchorage-dependent G1 checkpoint and replicate their DNA in suspension culture.
- Epithelial cells overexpressing c-Myc can down regulate the CDK inhibitor p27 and activate cyclin E-CDK2 in the absence of adhesion.

Reportable outcomes:

1. Poster presented at the 38th American Society for Cell Biology Annual Meeting, San Francisco, CA. December 1998.
   The role of c-myc in bypassing an anchorage-dependent G1 checkpoint in human mammary epithelial cells. C.M. Benaud and R.B.Dickson.

2. Abstract submitted for the 39th American Society for Cell Biology Annual Meeting, Washington, DC. December 1999.

   Regulation of c-Myc by β1 integrins in mammary epithelial cells. C.M. Benaud and R.B.Dickson .

Conclusions:

To date, most of the studies on anchorage-dependent growth regulation have been performed only on fibroblasts. Fibroblasts and epithelial cells have a different relationship with their ECM. Primary mammary epithelial cells have been shown to receive specific survival signals from basement membrane matrix which are not provided by attachment to plastic or type I collagen (17). Hence, epithelial cells may differ from fibroblasts in their interaction with the extracellular matrix, and the control of cell growth by the ECM may also vary.

Our results indicate that contrary to what has been described in fibroblasts, in mammary epithelial cells c-Myc expression is directly regulated by cell adhesion. In addition, we have shown that a β1 integrin-dependent signaling pathway can directly upregulate c-Myc expression and that both integrin and growth factors signaling pathways cooperate for maximal induction of c-Myc.

We have also shown that similarly to mesenchymal systems, mammary epithelial cell growth is controlled by an anchorage-dependent G1 checkpoint. Furthermore, deregulated expression of c-Myc can override this checkpoint by downregulating the CDK inhibitor p27, and activating the late G1 cyclin E-CDK2 complex. Taken together our results show that sustained expression of c-Myc, which is required for progression through the cell cycle, requires cell adhesion. Overexpression of c-Myc which often occurs in breast cancer, can unable human mammary epithelial cells to ignore the integrin control of Myc and progress through G1 phase and G1/S transition in an anchorage-independent fashion.

This study gives a light on the mechanism by which deregulated expression of c-Myc may unable mammary epithelial-derived cancer cells to proliferate in an uncontrolled manner, ignoring the regulatory environmental clues.

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Appendices:

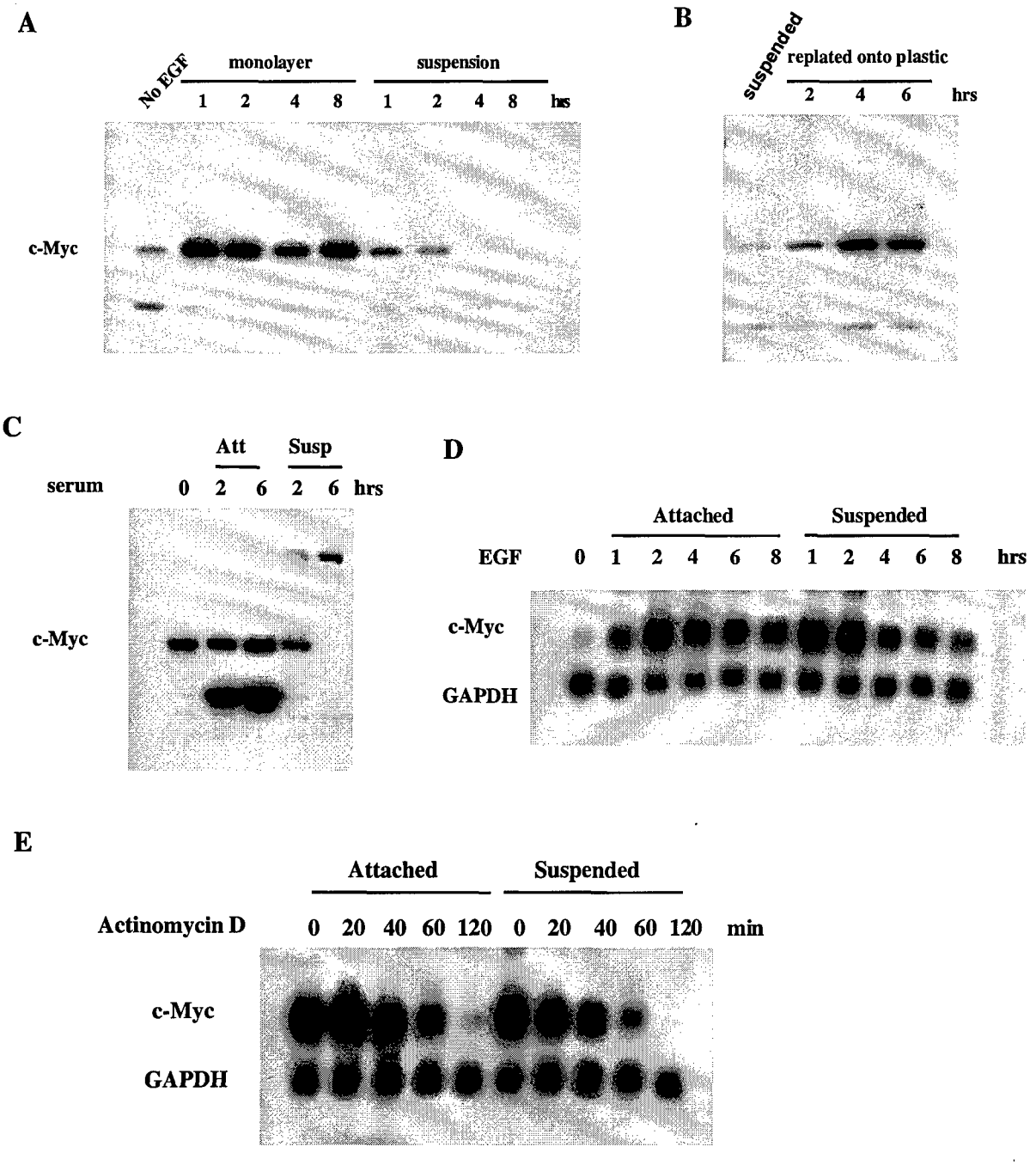

Figure 1: Regulation of c-Myc by cell adhesion. c-Myc western blot in adherent and non-adherent EGF stimulated A1N4 cells (A), serum stimulated MCF-10A (C) and following readhesion of suspension culture of A1N4 cells (C). Northern blot of Myc mRNA in adherent and non-adherent A1N4 cells at various times following EGF stimulation (D) and following Actinomycin D treatment of adherent and non-adherent A1N4 cells (E). Actinomycin D was administred 4 hours after EGF addition.

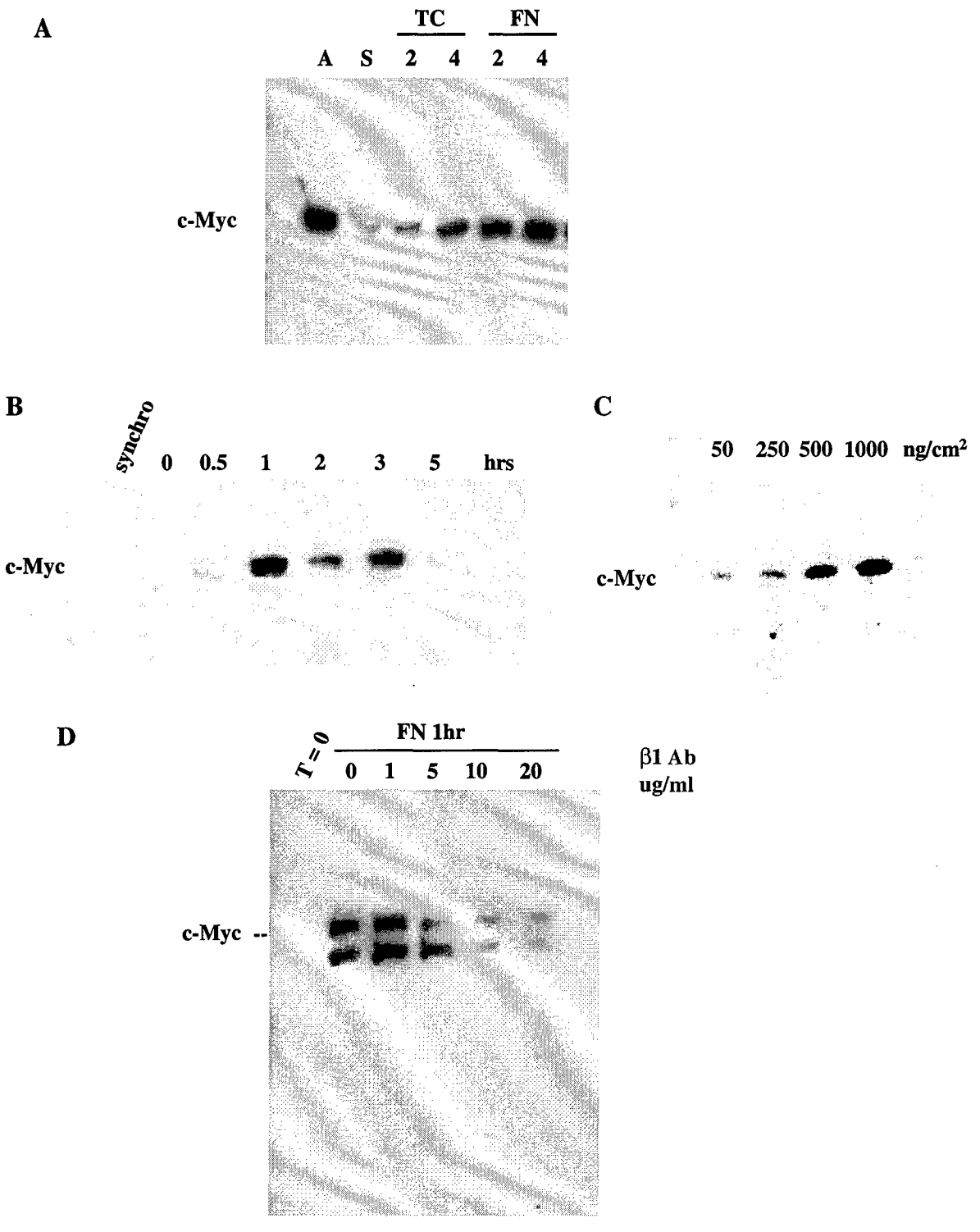

Figure 2: A-Western blot analysis of c-Myc comparing c-Myc induction, in the presence of EGF, following reattachement of suspension culture (S) either to tissue culture dish (TC) or to fibronectin coated dishe(Fn). B- Time course of the transient induction of Myc protein by adhesion to FN in the absence of growth factor addition. C- Fibronectin density-dependent induction of c-myc protein. D- Inhibition of fibronectin-induced Myc expression by β1 blocking antibody. B,C,and D were performed in the absence of exogenous growth factors.